The tumour suppressor gene \textit{WWOX} is mutated in autosomal recessive cerebellar ataxia with epilepsy and mental retardation

Martial Mallaret,1,2,* Matthis Synofzik,3,4,* Jaeho Lee,5 Cari A. Sagum,5 Muhammad Mahajnah,6,7 Rajech Sharkia,8 Nathalie Drouot,2 Mathilde Renaud,1,2 Fabrice A. C. Klein,2 Mathieu Anheim,1,2 Christine Tranchant,1,2 Cyril Mignot,9 Jean-Louis Mandel,2,10 Mark Bedford,5 Peter Bauer,11 Mustafa A. Salih,12 Rebecca Schüle,3,4,13 Ludger Schöls,3,4 C. Marcelo Aldaz5 and Michel Koenig2,10,14

1 Department of Neurology, Hôpital de Hautepierre, Strasbourg, France
2 Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), INSERM-U964/CNRS-UMR7104/Université de Strasbourg, Collège de France, 67404 Illkirch, France
3 Department of Neurodegenerative Diseases and Hertie-Institute for Clinical Brain Research, University of Tübingen, Germany
4 German Research Centre for Neurodegenerative Diseases (DZNE), Tübingen, Germany
5 Department of Molecular Carcinogenesis, University of Texas M. D. Anderson Cancer Centre, Smithville, TX, USA
6 Paediatric Neurology and Child Developmental Centre, Hillel Yaffe Medical Centre, Hadera 38100, Israel
7 Rappaport Faculty of Medicine, The Technion, Haifa 31096, Israel
8 The Triangle Regional Research and Development Centre, P. O. Box-2167, Kfar Qar’i-30075, Israel; Beit-Berl Academic College, Beit-Berl 44905, Israel
9 APHP, Groupe Hospitalier Pitié Salpêtrière, Unité Fonctionnelle de Génétique Médicale, Paris, France
10 Laboratoire de Diagnostic Génétique, Nouvel Hôpital Civil, 67091 Strasbourg, France
11 Institute of Medical Genetics and Applied Genomics, University of Tübingen, Germany
12 Division of Paediatric Neurology, Department of Paediatrics, College of Medicine, King Saud University, Riyadh, Saudi Arabia
13 Dr. John T. Macdonald Foundation Department of Human Genetics and John P. Hussman Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, FL 33136, USA
14 INSERM UMR_S 827, Laboratoire de Génétique Moléculaire, CHU de Montpellier, and Université de Montpellier 1, UFR de Médecine, 34000 Montpellier, France

*These authors contributed equally to this work.

Correspondence to: Dr Michel Koenig,
INSERM UMR_S 827,
Institut Universitaire de Recherche Clinique,
641 Avenue du Doyen Gaston Giraud,
34093 Montpellier cedex5, France
E-mail: michel.koenig@inserm.fr

We previously localized a new form of recessive ataxia with generalized tonic-clonic epilepsy and mental retardation to a 19 Mb interval in 16q21-q23 by homozygosity mapping of a large consanguineous Saudi Arabian family. We now report the identification by whole exome sequencing of the missense mutation changing proline 47 into threonine in the first WW domain of the WW domain containing oxidoreductase gene, \textit{WWOX}, located in the linkage interval. Proline 47 is a highly conserved residue that is part of the WW motif consensus sequence and is part of the hydrophobic core that stabilizes the WW fold. We demonstrate that proline 47 is a key amino acid essential for maintaining the \textit{WWOX} protein fully functional, with its mutation into a threonine resulting in a loss of peptide interaction for the first WW domain. We also identified another highly conserved...
Introduction

Autosomal recessive cerebellar ataxias are a clinically and genetically heterogeneous group of inherited neurodegenerative disorders that affect the cerebellum, the spino-cerebellar long tracts and often the peripheral nerves. Recessive ataxias may present as a pure cerebellar syndrome or are associated with neurological symptoms such as peripheral neuropathy, cognitive impairment, optic atrophy, hearing loss, seizures or extra neurological symptoms such as cardiomyopathy and diabetes mellitus in Friedreich’s ataxia (MIM# 229300; Anheim et al., 2012). Main clinical features are uncoordinated gait with frequent falls, upper limb coordination problems, impairment of speech, swallowing and eye movements.

Recessive ataxias associated with myoclonus, epilepsy and cognitive deterioration are classified within the progressive myoclonic epilepsies, and include Unverricht-Lundborg disease, Lafora disease, neuronal ceroid lipofuscinoses, sialidoses, action myoclonus-renal failure syndrome and Gaucher’s disease, suggesting a global CNS involvement. Mitochondrial inheritance in myoclonic epilepsy with ragged red fibres (MERRF, MIM# 545000) or autosomal dominant inheritance in dentato-rubro-pallido-luysian atrophy (DRPLA, MIM# 125370) may also be responsible for a progressive myoclonic epilepsy phenotype. Other recessive ataxias with a frequent occurrence of seizures, such as infantile onset spino-cerebellar ataxia (IOSCA, MIM# 271245) or recessive ataxia with coenzyme Q10 deficiency (ARCA2, MIM# 612016) are usually excluded from the group of progressive myoclonic epilepsies.

We reported a new form of childhood onset autosomal recessive cerebellar ataxia with generalized tonic-clonic epilepsy and mental retardation in a large consanguineous family from Saudi Arabia with four affected children (Gribaa et al., 2007; SCAR12, MIM# 614322). MRI of one patient revealed posterior white matter hyperintensities, whereas muscle biopsy showed vacuolization of the sarco-tubular system. The gene was localized by homozygosity mapping to a 19-Mb interval in 16q21-q23 between markers D16S3091 and D16S3050. We report here the identification by whole exome sequencing of the protein coding exons of WWOX (Santa Cruz biotechnology) (nine coding exons, with the same kit, but analysed with help of the GEnomes Management Application (GEM.app; Gonzalez et al., 2013). Search for mutations in genes known to be responsible for neurological phenotype including recessive cerebellar ataxia was performed and no mutation was found. Genomic DNA of 189 patients from 182 families with sporadic or recessive ataxias was screened by direct sequencing of the protein coding exons of WWOX (nine coding exons and an alternative exon 6).

WW domain three-dimensional modelling and western blot analysis

Protein sequence conservation was studied with the PipeAlign online software (http://bips.u-strasbg.fr/PipeAlign). Three-dimensional homology models of WW domains were generated with the online SWISS-MODEL software (http://swissmodel.expasy.org; Arnold et al., 2006), based on sequence alignment with the closest WW homologues found in the protein database (pdb), which were used as reference. Homology models and nuclear magnetic resonance model of WW domains were then superimposed using PyMol (http://pymol.org). Western blot analysis was performed with polyclonal IgG antibodies directed against the first 19 amino acids of WWOX (Santa Cruz biotechnology, dilution 1:500). Human skin fibroblasts were cultured from a skin biopsy from Patient II-3 and from four different controls in...
Dulbecco modified Eagle’s medium with glucose 1 g/l, 10% foetal calf serum and gentamicine, at 37°C with 5% CO2.

Construction of wild-type and mutant WW domain fusion proteins and peptide pull-down assays

To study WWOX protein–protein interaction in vitro, we used synthetic constructs with glutathione-S-transferase (GST) tags for affinity purification. GST fusion proteins containing WWOX first and second WW domains (WWOX1–2), and only the first WW domain (WWOX1) were constructed by insertion of PCR amplified fragments of the WWOX complementary DNA into the BamHI and EcoRI sites of the bacterial expression vector pGEX-2TK (Pharmacia). The resulting fusion proteins contain the WWOX amino acids 16 to 94 (GST-WWOX1–2) and amino acids 16 to 53 (GST-WWOX1) as previously described (Ludes-Meyers et al., 2004).

Site-directed mutagenesis was performed using the GeneArt Site-Directed Mutagenesis PLUS kit (Invitrogen). The target for mutation was p.Pro47Thr (c.139C>A) in the first WW domain of fusion proteins WWOX1–2 and WWOX1. All sequences were verified by DNA sequencing.

GST fusion proteins were purified from bacterial lysates through the use of glutathione sepharose beads (GE Healthcare). In vitro peptide pull-down assays were conducted by overnight pre-binding of 15 μg of the biotinylated WPB1 peptide (SGSGVTPPPPTVGGTVG) with 1 μg of GST fusion protein in 500 μl of binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl2) and rocking at 4°C. After pre-washing with binding buffer, 25 μl of streptavidin agarose beads (Millipore) were incubated with the peptide-protein mix and rocked for 1 h at 4°C. Agarose beads were then washed three times with binding buffer. 30 μl of 2× protein loading buffer was added to the agarose beads and boiled. Protein separation was performed by SDS-PAGE and analysed by western blot using a rabbit anti GST-specific primary antibody developed in M.B. lab (Espejo et al., 2002; 1:2000 dilution).

Animal experiments

All animal research was conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International at the University of Texas, M.D. Anderson Cancer Centre, following international guidelines and all research was specifically approved by the University of Texas M.D. Anderson Cancer Centre Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3343-01).

For the generation of Wwox knock-out mice, we crossed male Wwox<sup>fl/fl</sup> mice (129Sv/C57Bl/6 background) (Ludes-Meyers et al., 2009) with female Bk5-Cre mice (129Sv/C57Bl/6 background). Cre recombinase in these females is activated in oocytes with Cre protein persisting in the embryo leading to constitutive recombinase activity. Cre recombinase in these females is activated in oocytes with Cre protein persisting in the embryo leading to constitutive recombinase activity. Three-dimensional modelling of the first WW domain

We modelled the structure of the WW1 domain of WWOX according to the structure of its closest homologue NEDD4 WWIII (pdb code 1I5H) and compared it to the WW structures of transcription elongation regulator 1/TCERG1 (previously known as FBP28, pdb code 1EOL) and polyglutamate binding protein 1/QPBP1 (modelled according to the structure of PIN1, pdb code 3KAB.pdb) for which mutation of the equivalent proline or of the equivalent interacting tyrosine within the core, respectively.
Figure 1 Identification of WWOX mutations in the Saudi and Israeli Palestinian families. (A) Family trees showing consanguinity and segregation of the disease with mutations c.139C>A, causing the p.Pro47Thr missense change (47T) and c.1114G>C causing the p.Gly372Arg missense change (372R). (B) Sanger sequencing of the mutations c.139C>A and c.1114G>C: index patients are homozygous for the substitution and carrier relatives (mother I-2 in Family 1 and father I-1 in Family 2) are heterozygous for the same substitution. (C) Sequence comparison of the WW1 domain (left) and of the C-terminal part of the dehydrogenase/reductase domain (right) of WWOX from different species. Amino acids that are identical to the human sequence are shown in bold. The highly conserved aromatic residues that are part of the hydrophobic core of the WW1 domain are underlined. The mutant amino acids (threonine, T, and arginine, R), on top of the mutated amino acids (proline, P, and glycine, G, respectively), are indicated by an arrow. The mutated amino acids are conserved in all metazoan species analysed, except glycine 372 in some insects (endopterygota branch). Hs = human; Mm = opossum; Gg = chicken; Xt = frog; Dr = bony fish; Dm = fly; Ae = ant; Phc = louse; Cg = oyster; Hm = hydra; Nv = sea anemone; Ta = trichoplax (multi-cellular amoeba).
are known to dramatically destabilize the fold (Petrovich et al., 2006; Tapia et al., 2010). The position of WWOX proline 47 and tyrosine 34 precisely match the position of TCERG1 and PQBP1 equivalents, which are direct hydrophobic core neighbours and are crucial for proper folding of the WW domain (Fig. 2). Thus, the change of proline 47 into a threonine most likely destabilizes the WW1 domain.

Interaction studies of the mutant WW domain

To test this hypothesis, we constructed GST-WWOX fusion proteins containing the first WW domain (WWOX1) as well as both WW domains (WWOX1–2). We generated both wild-type and mutant versions [p.Pro47Thr (c.139C>A)] of these fusion proteins. As previously demonstrated, the first WW domain of WWOX represents a typical Group I WW domain with predilection for binding proline-proline-X-tyrosine motifs, where X stands for any amino acid, whereas no ligand was identified for the second WW domain (Ludes-Meyers et al., 2004). We performed peptide pull-down assays using as ligand a biotinylated oligopeptide from the WW domain-binding protein 1 (WBP1), a typical WW group I binder, containing a proline-proline-proline-tyrosine motif (PPPY). As can be observed in Fig. 3, the wild-type WWOX1 and WWOX1–2 fusion proteins were successfully pulled down whereas the p.Pro47Thr mutant versions failed to interact with the PPPY containing oligopeptide.

The high conservation of the proline 47 residue and the above experiments demonstrate the crucial role of this residue in WW domains, indicating that the observed mutation likely plays a key structural role leading to unfolding and dysfunction of WWOX1. This evidence suggests that p.Pro47Thr is the disease causing mutation in the ataxia/epilepsy family from Saudi Arabia.

Furthermore, western blot analysis from patient fibroblasts showed a similar level of WWOX protein compared to four different controls (Fig. 2) suggesting that the mutation does not alter global protein levels. Globally unchanged protein levels were also reported for the equivalent Tyr34 mutation of polyglutamine tract binding-protein 1 (PQBP1) involved in X-linked mental retardation disorders such as Golabi-Ito-Hall syndrome, showing that a disease causing mutation that affects the fold and the function of a WW domain does not necessarily result in enhanced mutant protein degradation (Tapia et al., 2010).

Mutation analysis of Family 2

After the identification and characterization of the first disease-associated WWOX mutation, a second mutation was identified by exome sequencing of a consanguineous Palestinian family. The two patients from this Israeli Palestinian family carried a highly conserved homozygous WWOX mutation (p.Gly372Arg;...
c.1114G>C in exon 9) (for conservation data and pedigree, see Fig. 1) which was not observed in dbSNP 137 or in the NHLBI 6500 exome variant server. The homozygous variant segregated with disease in the affected siblings and testing of the parents demonstrated localization in trans. The phenotype in this second family closely resembled the index family: both affected siblings showed an early disease onset in the first 2 years of life with generalized tonic-clonic epilepsy, mental retardation, and ataxia. In addition, prominent upper motor neuron affection with leg spasticity and positive bilateral extensor planatar response was present, thus resembling spastic ataxia or complicated hereditary spastic paraplegia. The mutated glycine 372 to threonine is sufficient to abrogate the affinity of WWOX for PPXY motif containing interacting partners, presumably by destabilizing the WW1 fold. Bottom: red ponceau stain of the pull-down blot (oligopeptide range), to demonstrate incubation with equal amounts of biotinylated WBP1 peptide.

### Conditional knock-out mouse model

As it seems that ataxia/epilepsy associated with WWOX mutation is very rare in humans, we sought to confirm the involvement of WWOX in neurodegeneration from the study of our mice Wwox knock-out model (Ludes-Meyers et al., 2009). These knock-out mice are characterized by a short lifespan of only 3 to 4 weeks maximum (Aqeilan et al., 2008; Ludes-Meyers et al., 2009). Interestingly, we observed that the Wwox knock-out mice start having a few spontaneous seizures at ~2 weeks of age. To investigate susceptibility to epilepsy, 16-day-old knock-out mice were exposed to sustained sound (11–14 kHz tone) for 5–10 min. A few knock-out mice (three of eight) presented with audiogenic tonic-clonic seizures in the first minutes after sound exposure. Seizures began with wild running and jumping, then progressed to tonic contraction of limbs and tail and often presented with limb clonic movements, followed by lethargy. At 20 days, the four surviving mice were exposed to a 14 kHz tone. All knock-out mice presented at different times with seizures, consisting as before of wild running followed by tonic contractions and clonic movements, and had uncontrolled sphincter relaxation (Fig. 4 and Supplementary Video 1). At the end of seizures, the mice presented with lethargy. Other stimuli such as animal handling also induced seizures on some occasions. At this point, all knock-out mice presented with balance disturbances when challenged to walk on the edge of the cage but they showed no pathological clasping reflexes. They eventually died before 4 weeks of age from failure to thrive. No wild-type mice of matched age and background (n = 8) presented with seizures upon 11 or 14 kHz sound exposure.

### Discussion

We describe the first germline mutations in WWOX causing a recessive form of early onset neurodegenerative disease in man including epilepsy, mental retardation, cerebellar ataxia, and—at least in the second family—prominent spasticity. WWOX was identified by Bednarek et al. (2000) and codes for a 414 amino acid protein containing two WW domains and a short-chain dehydrogenase/reductase (SDR) domain (suggesting a role in steroid metabolism). WWOX is an ubiquitous protein with high expression in prostate, gonads, breast, lung, endocrine tissues, cerebellum and brain (Nunez et al., 2006; Aqeilan et al., 2007). Wwox is expressed in mouse developing nervous system including cerebral cortex and cerebellum from Day 12–14 in utero to adulthood (Chen et al., 2004). We demonstrate that the WWOX mutation, p.Pro47Thr, which could be modelled by our in silico model, renders WWOX unable to bind protein partners through the WW1 domain, and that the complete loss of function of WWOX in mice results in epilepsy and balance disturbances, phenotypes reminiscent of the clinical presentation of all our affected patients with WWOX mutations. The Wwox knock-out mice show a progressive onset ataxia associated with epilepsy, 67 patients with onset of ataxia before 3 years, and 135 patients with onset of ataxia before 20 years) revealed no additional mutation.
susceptibility to spontaneous and audiogenic tonic-clonic epilepsy, suggesting a neurodegenerative process. However, the severe condition and early death of the Wwox knock-out mice prevented us from testing whether the balance problems were directly related to cerebellar dysfunction or not. Interestingly, a spontaneous rat mutation of Wwox, lde (lethal dwarfism and epilepsy) results in a condition similar to the presentation of the mutant WWOX patients and the Wwox knock-out mice, as homozygous

Figure 4 Still frames of audiogenic tonic-clonic seizures of Wwok knock-out mouse. Twenty-day-old Wwok knock-out mice were exposed for 10 min to a digital 14 kHz tone. After 30 s sound exposure, the first mouse presented a wild-running phase (see Supplementary Video 1), followed by tonic contractions that lasted over 42 s (A). After 4 min sound exposure, two other mice also experienced clonic movements (B and Supplementary Video 1).
Idê rats present with ataxic gait and audiogenic seizures (Suzuki et al., 2007, 2009). The Idê mutation is a 13 bp deletion in exon 9 leading to a frameshift of the last 44 codons of Wwox and replacement with a novel open reading frame of 54 codons. Mutated WWOX is not detected in western blots of Idê rat tissues. The milder presentation of the human disease compared with the mouse and rat models is presumably because of a partial loss of function mutation as the mutant p.Pro47Thr WWOX protein is still present, at least in human skin fibroblasts, and the dehydrogenase/reductase domain of the mutant protein is presumably still functional in Family 1 and partially functional in Family 2, unlike the dehydrogenase/reductase domain of the WWOX mouse and rat models. Homozygous complete loss of function of WWOX in humans may also cause early death or embryonic lethality, suggesting that WWOX related ataxia cases are rare, in line with our failure to identify additional WWOX mutation in a large cohort of patients with ataxia.

WWOX is a cytoplasmic protein that participates in a number of cellular processes including growth, differentiation and tumour suppression. WWOX somatic mutations, including homozygous deletions, have been involved in different human cancer cell types (Paige et al., 2001). WW domains are protein-protein interaction domains that recognize proline-rich sequences. Numerous interactors of the WW1 domain of WWOX have been identified, including p73, RUNX2, ERBB4, Dvl2, SIMPLE/LITAF among others (Agelian and Croce, 2007). Alteration of WW1 is responsible for a loss of interaction with Dvl2 (Bouteille et al., 2009). Direct mutagenesis of amino acids 44 to 47 of the WW1 domain of WWOX leads to a loss of interaction with SIMPLE/LITAF (Ludes-Meyers et al., 2004), a protein mutated in a dominant form of Charcot–Marie–Tooth disease, CMT1C (Street et al., 2003). Heterozygous deletion of exons 6 to 8 (amino acids 173 to 352 of WWOX) has recently been described in a single patient with ambiguous genitalia having a 46,XY disorder of sex development (White et al., 2012). The deletion is not causing a frame-shift but leads to a smaller protein missing part of the SDR domain. It is possible that the heterozygous deletion of exons 6 to 8 of WWOX is coincidental in the patient with ambiguous genitalia, as no such phenotype is present in heterozygous animal models or in the parents and carrier siblings of the Saudi or Palestinian patients. Future research should investigate the presence of WWOX variants in patients with epilepsy, mental retardation, and/or complicated hereditary spastic paraplegia (CHSP), as the phenotype of our affected subjects indicates that these might also be presenting features of WWOX patients. Analysis of larger paediatric cohorts of early onset ataxia and/or generalized epilepsy and mental retardation should allow us to evaluate the prevalence of WWOX mutations and to perform genotype-phenotype correlation studies.

Disease mechanisms resulting from WWOX mutations are difficult to infer because the WWOX protein has numerous functions and protein partners. Moreover, the potential substrates of the WWOX dehydrogenase domain are not known. WWOX has been described as a tumour suppressor gene. Interestingly, neither the patients (current age ranging from 17 to 26 years in Family 1, and 5 and 10 years in Family 2) nor the heterozygous carriers presented with tumour development, but we cannot exclude that the patients may present an increased risk of developing cancer at a later stage. Further studies are needed to establish the full spectrum of diseases resulting from WWOX alterations.

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Supplementary material

Supplementary material is available at Brain online.

Web Resources

UCSC Genome Browser: http://genome.ucsc.edu/index.html
Ensembl Genome Browser: http://www.ensembl.org/index.html
Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA(http://evs.gs.washington.edu/EVS) [June, 2013]
SplicePort software: http://spliceport.ccb.ufl.edu
PipeAlign software: http://bips.u-strasbg.fr/pipeline
PyMol software: http://pymol.org

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